ON-CHIP MULTIPLEX PCR AMPLIFICATION DIRECTLY FROM WHOLE BLOOD

R. S. Wiederkehr[1,2], B. Jones[1], S Peeters[1],T Stakenborg[1], O. Ibrahim[3,4], P Fiorini[1], H Tanaka[5], I. Yamashita[5] T. Matsuno[5] and L. Lagae[1,2]

[1]*Life Science Technologies Department, IMEC, Leuven, Belgium*
[2]*Department of Physics and Astronomy, KU Leuven, Leuven, Belgium*
[3]*Mechanical Engineering Department, Alexandria University, Alexandria, Egypt*
[4]*KACST-Intel Consortium Centre of Excellence for Nano-manufacturing Applications (CENA), Riyadh, KSA*
[5]*Advanced Technology Research Laboratory, Panasonic Corporation, Kyoto, Japan*

ABSTRACT

A multiplex polymerase chain reaction (PCR) direct from human blood using a silicon/glass microfluidic chip is demonstrated. The microfluidic chip consists of a mixer (for combining whole blood and reagents), a microreaction chamber (for cell lysis and PCR thermal cycling) and a micropillar filter (for purification of the amplified DNA). The reported microsystem can serve as a compact DNA amplification module for use in a larger lab-on-chip microsystem. The device was demonstrated by successfully amplifying three fragments of different length (142, 105 and 72 base pairs) directly from whole blood. The influence of surface coating and polymerase enzyme on the microsystem functionality is reported.

KEYWORDS: DNA amplification, microfluidics, Single-Nucleotide-Polymorphism (SNP), multiplex PCR

INTRODUCTION

In recent years, significant improvements have been made in the development of micro total analysis systems (μTAS) for point of care testing [1]. The use of such microdevices offers several distinct advantages such as short assay times, low reagent consumption, as well as the potential to integrate multiple processing steps (e.g., sample preparation, DNA amplification, detection, etc.). PCR is the most popular method to analyze DNA for a large variety of life science or clinical diagnostic applications. Thus, scaling PCR to minute volumes compatible with a μTAS has received great attention. However, a potential complication to this down scaling and microsystem integration is the significant increase in surface area to volume ratio of the microreaction chamber, which may introduce some inhibiting effects to the PCR. For instance, polymerase adsorption to the reactor walls has been theorized to decrease the PCR reaction efficiency [2]. Hence, successful amplification experiments in microsystems require a careful choice of the reagents and often dedicated surface treatments. The reaction is more easily controlled if purified DNA is used instead of untreated biological fluids. In this work, we describe a protocol used to obtain successful on-chip amplification results directly from whole human blood.

EXPERIMENTAL

The device used in this paper is presented in Figure 1. More details on the fabrication process have been described before [3]. Briefly, the fluidic structures were etched in silicon by deep reactive ion etch and sealed with Pyrex by anodic bonding, while fluidic access were provided by backside etch of silicon. The device is composed of three elements, a mixer, a microreactor and a filter. The mixer is designed to rely on molecular diffusion in a serpentined channel about 55 mm long, whose width and depth are 100 μm and 200 μm, respectively. The reaction chamber occupies a region of 3.4 x 3.4 mm^2 and its total volume is about 2 μl. It has also a meandered shape, without sharp concave angles. This design helps avoid air bubble entrapment during filling. Effective thermal insulation of the reaction chamber is realized by etching trenches into the high thermal conductivity silicon surrounding the cavity, leaving only thin channels, which wind around the cavity, for the fluidic connection. Thanks to this design, the thermal mass of the microreactor is very small and fast temperature cycling is achieved, with transient times between typical PCR temperatures steps well below 2 seconds [3].

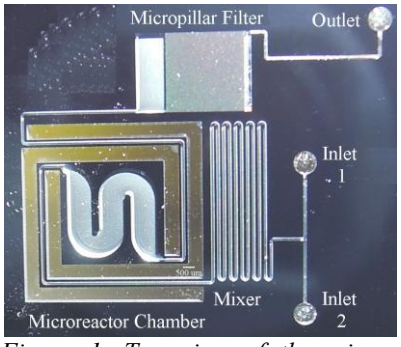


Figure 1. Top view of the micro-device, which consists of a mixer, a reactor, and a micropillar purification filter. The total area of the heated chamber is 3.4x3.4 mm^2

The microreactor temperature is controlled by a commercially-available, microfabricated thermoelectric cooler attached to the backside of the device. Depending on the direction of the current, this element acts as a heater or a cooler. In the cooling phase the heat extracted by the thermoelectric element is evacuated to the ambient through a pin fin heat sink (not shown).

The filter is formed by an array of micropillar with a diameter of 25 μm and an interpillar distance of 5 μm. This filter retains large size molecular debris while allowing amplified DNA to pass through it towards the outlet of the microdevice. More details on the fabrication of such a filter has been described before [4].

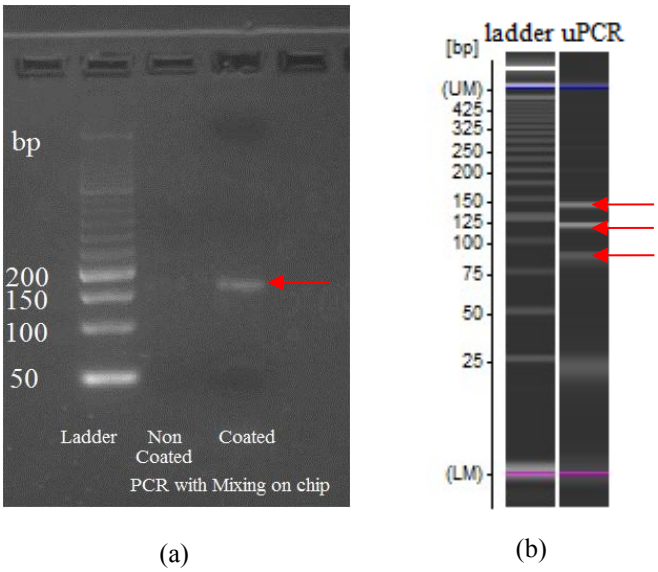
For some initial singleplex PCR experiments, the chips were used directly after cleanroom processing with no further surface treatments. For

other singleplex PCR as well as the multiplex PCR tests, the surface was silanized by injecting a solution of 0.005 % (v/v) octadecyltricholosilane dissolved in toluene at a flow rate of 10 μL/min for 20 minutes. After coating, the device was flushed with isopropanol for 20 minutes and dried in an oven at 100°C for 3 hours.

Initial singleplex experiments were performed in order to optimize the protocol, which was later applied to multiplex amplification. The protocol for the singleplex amplification was first tested in a conventional PCR tool. Two different assays designed for direct amplification from whole human blood were tested: (1) Titanium Taq (Clontec Laboratories) with EzWay™ buffer (Komabiotech) and (2) KOD Xtreme™ hot start (Merck Millipore) with KOD extreme buffer. The reaction conditions are given in Table 1. In all experiments blood and PCR reagents were introduced separately through the two mixer inlets and amplified in the microreactor chamber. The expected amplicon size for the singleplex reaction is 142 base pairs. For the multiplex amplification, the optimized conditions for the singleplex reaction were used and two more primer pairs were added, whose sequences are given in Table 1. These primers select two DNA segments of 105 and 72 base pairs long. After the amplification step, the solution was filtered using the on-chip micropillar array. The filtrate was then extracted from the chip and analyzed by either slab gel or capillary electrophoresis.

Table 1. PCR protocols used to amplify DNA from whole blood.

Compound and concentration	**Volume (μl)**		
	Singleplex		**Multiplex**
2X KOD Extreme Buffer	**1**	**---**	**1**
Ez Way Buffer	**---**	**0.4**	**---**
dNTPs (2 mM)	**0.2**	**0.2**	**0.2**
Forward Primer 1 (10 μM) *ATTGTAGGGAAATGTCTGTCTGAT*	**0.2**	**0.2**	**0.1**
Reverse Primer 1 (10 μM) *ACACCAATCTCTACATCATAAGGAG*	**0.2**	**0.2**	**0.1**
Forward Primer 2 (10 μM) *CACCTCTGACTTCTCAGGTGT*	**---**	**---**	**0.1**
Reverse Primer 2 (10 μM) *GCCTCTAACATTCTGTTTAGGAGA*	**---**	**---**	**0.1**
Forward Primer 3 (10 μM) *TCCCGTCACCTGAAACTGCTGTCACC*	**---**	**---**	**0.1**
Rerverse Primer 3 (10 μM) *GCATATTTGGTGGAAAAGTCTACAG*	**---**	**---**	**0.1**
KOD Polymerase	**0.08**	**---**	**0.04**
Titanium Taq Polymerase	**---**	**0.04**	**---**
Whole Blood	**0.2**	**0.2**	**0.16**
DI Water	**0.12**	**0.76**	**---**
Total Volume (μl)	**2**	**2**	**2**
Temperature Cycling performed during PCR 98°C – 120s cell lysis, 98°C-10s denaturation 62oC -10s annealing 68oC- 10s extension 30x			



(a) (b)

Figure 2. Gel electrophoresis results of the singleplex PCR amplification (a) and capillary electrophoresis results for the multiplex PCR reaction (b) using coated chips. Arrows point to the amplified bands.

RESULTS AND DISCUSSION

For initial PCR optimization, two different polymerases were tested to amplify a 142 base pair fragment. For both cases, amplification of the targeted DNA fragment was observed when amplified using the conventional PCR tool. The process was then transferred to the chip and repeated several times. In most cases, no or only non-specific amplification was observed. This irreproducibility could have multiple origins, but is most likely due to a non-controlled interaction of the polymerase or other reagents with the surface of the fluidic chip or a non-effective mixing of the sample with the reagents. To test the initial assumption, chips were coated with octadecyltricholosilane aiming to reduce non-specific adsorption. Indeed after coating the chips, reproducible and stable results were obtained at least for the KOD polymerase. The gel electrophoresis results are shown in Figure 2a. In contrast, using the Titanium Taq polymerase with EzWay™ buffer, no successful on chip amplification could be obtained. The reasons for this are not entirely clear, but perhaps a different coating is needed for successful on-chip amplification. This observation confirms once more the complexity of the interaction of polymerase enzymes with the silicon surface, probably complicated by the effect of the associated buffer used. In absence of a clear understanding of this interaction, the choice of the best suited polymerase and buffer can only be made by means of a trial and error process.

The optimized protocol for on-chip PCR direct from blood is listed in Table 1. Once the singleplex reaction was optimized, a multiplex reaction was tested using KOD as an enzyme and using the same reaction conditions. As indicated by capillary electrophoresis measurements shown in Figure. 2b, all three genomic fragments were successfully amplified directly from blood. This strongly indicates that our optimized on-chip PCR is robust and likely other assays optimized on a conventional tool using similar reagents can be transferred directly on-chip.

SUMMARY AND CONCLUSIONS

We demonstrated a simultaneous PCR amplification of three distinct DNA fragments directly from human whole blood in a 2 µl silicon-based micro reactor with an integrated mixing module. The inhibiting effect due to surface interactions between the reagents and silicon surface was decreased by coating the surface prior to amplification. The suitability of two different polymerases, KOD and Titanium Taq polymerase, was investigated. While both assays gave satisfactory amplifications directly from blood using the conventional PCR tool, only the KOD polymerase gave positive results on-chip. The ability to amplify genomic fragments directly from blood on a small, disposable chip without the need of additional sample preparation steps may be a strong enabler for future DNA diagnostic applications in a point-of-care setting. For this reason, current efforts are being made towards integrating the thermal sensors and heaters as well as detection on-chip as well.

REFERENCES

[1] "Microfluidics and point of care diagnostics" S. K. Sia and L.J. Kricka, Lab on a Chip, **8**, 1982 (2008).

[2] "Biochemical analysis and optimization of inhibition and adsorption phenomena in glass-silicon PCR chip", I. Erill *et al.*, Sensors and Actuators B, **96**, 685 (2003).

[3] "Silicon Based System for Single-Nucleotide-Polymorphism Detection: Chip Fabrication and Thermal Characterization of Polymerase Chain Reaction Microchamber", B. Majeed *et al.*, Japanese Journal of Applied Physics, **51**, 04DL01 (2012).

[4] "An Integrated Microsystem for Allele-Specific PCR Amplification of Genomic DNA Directly from Human Blood", B. Jones *et al.*, Proceedings of the 16th Int. Conf. on Miniaturized Systems for Chemistry and Life Science, 611 (2012).

CONTACT

R.S. Wiederkehr, Tel: +32 16 288669, e-mail: wiederk@imec.be